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Rapid *in vitro* Multiplication of Sugarcane Elite Genotypes and Detection of Sugarcane Mosaic Virus through Two Steps RT-PCR

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ABSTRACT

A voluminous body of research has reported the establishment of efficient protocols for sugarcane multiplication through tissue culture. However, a reliable and reproducible *in vitro* plant production system remains obscured. Furthermore, validation of virus free nature of *in vitro* plants using molecular techniques is the most challenging one. Considering the need for high yielding cultivars due to land and constraints, this study was devised for mass multiplication of high yielding elite cultivars of sugarcane viz. HSF-240, YT-55 and YT-53. Use of 100% Clorox for surface sterilization of apical and lateral buds, and of cefotaxime (500 mg L^{-1}) for controlling bacterial contaminants revealed complete sterilization of field grown explants. Culture initiation was dependent upon plant growth regulators (PGRs), genotype and type of explants. The highest shoot initiation frequency of 96% was obtained with combination of four plant growth regulators (0.1 mg L^{-1} BAP), (0.1 mg L^{-1} NAA), (0.1 mg L^{-1} Kn) and (0.1 mg L^{-1} GA_3). Maximum shoot number (17.4) was exhibited by HSF-240 on MS media when the concentrations of BAP, Kn and GA_3 were increased to 1 mg L^{-1} in combination with NAA (0.25 mg L^{-1}) indicating preference for higher concentrations of PGRs. Half-strength MS media with 6% sucrose resulted in increased root length (9.2 cm) and root number (20.5) Hardening efficiency of 98.6% was achievable in sandy clay loam soil. Two steps reverse transcription PCR (RT-PCR) was successfully employed for detection of sugarcane mosaic virus (SCMV) in *in vitro* plants. These results have implications for understanding optimum conditions for *in vitro* mass production of sugarcane plants, molecular detection of SCMV in *in vitro* raised plants, and stable genetic transformation studies.

Key Words: Sugarcane; Tissue culture; Plant growth regulators; Micropropagation; Sugarcane mosaic virus; RT-PCR
Abbreviations: BAP: 6-Benzylaminopurine; NAA: Naphthalene acetic acid; Kn: Kinetin; GA_3: Gibberellic acid; SCMV: Sugarcane Mosaic Virus; RT-PCR: Reverse Transcription Polymerase Chain Reaction

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) belongs to Poaceae family, and has been widely cultivated on tropical and subtropical regions globally. It is a high valued cash crop and exclusive source of 75% world sugar production (Lakshmanan *et al.*, 2006). This crop provides many by-products for bio-factory such as alcohol, butanol, acetic acid, animal feed and paper besides, sugar and energy (Garcia *et al.*, 2007). Sugarcane is genetically a complex crop that possesses highly variable chromosome number (octaploid; x = 10; 8x = 80). Being highly cross pollinated in nature, this crop requires specific, hot and humid climate for flowering (Gill *et al.*, 2006). The area under cultivation and yield of this crop is becoming stagnant over the years. Hence it is desirable to sustain the yield without expansion in area. For this purpose, introduction of high yielding disease and virus free varieties with genetically improved traits in short period of time is the best strategy. Micropropagation bears high potential for rapid clonal

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propagation of this vegetatively propagated crop. However, a reliable and reproducible *in vitro* plant production system remains enigmatic.

Tissue culturing of sugarcane has acquired significant attention because cane is now considered as an important cash crop from economical viewpoint. Initially, the *in vitro* regeneration of sugarcane was executed by Nickel (1964) and Heinz and Mee (1969). Moreover, tissue culture produces mass production of pathogen free plant stock and also offers an opportunity for commercial propagation of sugarcane in some countries including India, United States, Brazil and Cuba (Lakshmanan *et al.,* 2006).

The most commonly used methods of sugarcane micropropagation are shoot tip culture (Burner & Grisham, 1995; Lee, 1987) and callus culture (Ho & Vasil, 1983; Liu, 1993). The use of callus induction based system often results in genetic instability that leads to somaclonal variations. Therefore, callus phase is not desirable during micropropagation (Gill *et al.,* 2006) for sugarcane improvement. Moreover, efficient protocols for *in vitro* micropropagation and transformation are the landmarks for successful transgenic plants generation (Misra & Misra, 1993; Sharma &Vanamala, 2000; Iqbal *et al.,* 2011). Best known for their effects on shoot elongation the exogenous cytokinins are important for plant organogenesis though, micropropagation is genotype dependent.

A significant amount of cane production is lost because of many biological pests like virus. There is no chemical agent to eliminate virus from infected plants. In sugarcane, there are five viral infections viz. mosaic, sereh, streak, ratoon stunting and Fiji (Slykhuis, 1976). Among these viruses, sugarcane mosaic virus (SCMV) is the most widely distributed and resulted in mosaic disease (Koike & Gillaspie, 1989). It is found in almost all the cultivars of sugarcane (Gemechu *et al.,* 2006; Oertel *et al.,* 1997; Xie *et al.,* 2009). The crop yield is significantly reduced (10–22%) when incidence of infection level reaches to 50% (Handojo *et al.,* 1978). SCMV has been reported to be prevalent in more than 70 countries (Jeffery *et al.,* 1998). The initial symptoms of the mosaics disease are the chlorosis and yellowing of the green tissue like leaf followed by reddening and finally resulted in necrosis. It is difficult to get rid of these viruses and incipient diseases in field conditions. Hence, rapid multiplication of virus free *in vitro* sugarcane plants is indispensible. Therefore, the present study was envisaged to contribute to development of reproducible protocols for mass multiplication of elite cultivars of sugarcane and validation of their virus free nature using RT-PCR technique.

Based on tissue culture data we posit that culture initiation and multiplication, to a greater extent, are dependent on the type and genotype of explants as well as the type of cytokinins and their concentration. Moreover, two steps RT-PCR is an efficient strategy for the detection of SCMV in field grown and *in vitro* raised plants.

MATERIALS AND METHODS

Selection of sugarcane genotypes and culture conditions: All the experiments were performed at National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre (NARC), Islamabad, Pakistan. Fresh stems of the sugarcane cultivar HSF-240 were obtained from the fields of Sugarcane Program at National Agricultural Research Centre, Islamabad, Pakistan while that of YT-55 and YT-53 (Chinese origin) were acquired from the fields of National Sugar Crops Research Institute (NSCRI), Thatta, Sindh, Pakistan. Apical buds ranging in size from 2 to 5 mm and axillary buds with size of 3mm in thickness and 4–8 mm in diameter were excised from the stems. Explants were cut with a sharp scalpel and washed with detergent to remove the dust particles. For surface sterilization, Clorox (commercial bleach comprising 5.25% v/v sodium hypochlorite; an active agent) at 70% and 100% concentrations with 20 and 10 min of time duration was applied under aseptic conditions.

Without a special note Murashige and Skoog (1962) medium was used throughout the study for all the regeneration, shoot induction, shoot multiplication and root induction experiments. All the experiments were repeated three times for biological as well as technical replicates. MS mineral media with B5 vitamins supplemented with 3% sucrose and solidified at 0.2% phytagel was used. The pH of the media was adjusted at 5.7 ± 0.1 before autoclaving. All the plant growth regulators (PGRs) and antibiotic (cefotaxime @ 500 mg L^{-1}) were incorporated in the autoclaved media after sterilizing with 0.22 µm millipore filters. Cultures were incubated at a temperature of 25±1ºC under 16 h of photoperiod at light intensity of 2200 lux.

Culture initiation, multiplication and root induction: Considering that shoot induction is the crucial step during tissue culture of any crop, three types of plant regulators namely auxins (NAA), cytokinins (BAP and Kn) and gibberellin (GA_3) in three different media combinations (SI_1, 0.1 mg L^{-1} GA_3; SI_2, 0.1 mg L^{-1} Kn; SI_3, 0.1 mg L^{-1} BAP+ 0.1 mg L^{-1} Kn+ 0.1 mg L^{-1} GA_3+ 0.1 mg L^{-1} NAA) were employed. The sterilized explants were shifted to shoot induction media (SI_1, SI_2, SI_3) under aseptic conditions. After a period of 21-25 days emerged shoots of ≥4 cm in length were transferred to shoot multiplication media (SM_1, SM_2).

The shoot multiplication of *in vitro* cultures is an important step in the regeneration of any crop for commercial exploitation, where high and the most rapid multiplication is required. Therefore, four different plant regulators (NAA, BAP, Kn & GA_3) in various concentrations with two different MS media combinations (SM_1: 0.5mg L^{-1} BAP + 0.5 mg L^{-1} Kn + 0.5 mg L^{-1} GA_3+ 0.1 mg L^{-1} NAA; SM_2: 1 mg L^{-1} BAP + 1 mg L^{-1} Kn + 1 mg L^{-1} GA_3 + 0.25 mg L^{-1} NAA) were adopted for multiplication. The shoots emerged during shoot induction were transferred to shoot multiplication media. The cultures

were incubated for 25–30 days to achieve the optimal plant regulators requirement for maximum frequency of shoot multiplication.

To evaluate the effect of plant hormones on each genotype, half strength MS salt with B5 vitamins was employed without any PGRs (RI_1; MS + 0.5 mg L^{-1} NAA+ 0.5 mg L^{-1} IAA). The root induction response was further ascertained by utilizing plant hormones (NAA+IAA) in MS mineral media with B5 vitamins (RI_2; MS + 0.5 mg L^{-1} NAA+ 0.5 mg L^{-1} IAA). Healthy shoots were excised from the cluster of the shoots and placed on root induction media. The cultures were incubated for 4–5 weeks. The rooted plantlets were then transferred to sandy clay loam soil for hardening purpose under glasshouse conditions.

Molecular analysis for validation of virus-free nature of regenerated plants: Total RNA from leaf tissues of *in vitro* regenerated plants as well as from SCMV infected sugarcane plant was extracted using Pure Link™ RNA Mini kit (Invitrogen, Catalog # 12183-018A) according to manufacturer instructions. High quality RNA was used as template for the synthesis of first strand cDNA using the RevertAid™ Reverse Transcriptase kit (Fermentas, Catalog # EP0442). The sequence of SCMV coat protein was retrieved from NCBI database. Two pairs of gene primers for the amplification of SCMV coat protein (EU650180) were designed manually. The sequence forward primer of first pair (PF1/PR1) was 5′-GATGCAGGCGCTCAAGGAGGAGGTGG-3′ and that of reverse primer was 5′-GGTGAGATTTCGCTGAAGTCCATATCGT-3′. The expected size of the target product was 718 bp. The forward and reverse primer sequences of nested primer pair (PF2/PR2) were 5′-GAGGAGTTTGATAGGTGGTATGAA-3′ and 5′-CCATTAAACCACTCATGACAACTGTC-3′, respectively. This primer pair could amplify a product of 456bp.

Complementary DNA was used as template for RT-PCR analysis using Taq polymerase (Fermentas, catalogue # EP0402) according to manufacturer instructions. Each PCR reaction was performed in a 25 µL (final volume) of reaction mixture consisting of 2.5 µL 10X PCR buffer, 1 µL 25 mM $MgCl_2$, 0.5 µL 10 mM dNTPs, 0.5 µL 5 μL^{-1} Taq polymerase, 1 µL 10 pg forward primer, 1 µL 10 pg reverse primer, 2 µL 50 ng cDNA template and 16.5 µL double distilled water. The samples were subjected to 40 cycles of 1 min melting at 94 °C, 1 min annealing at 52°C for sugarcane mosaic virus coat protein gene and 1 min extension at 72°C followed by another 7 min final extension at 72°C. The amplified products were run on 2% agarose gel stained with ethidium bromide (0.5 µg mL^{-1}) and visualized and photographed under ultraviolet light. The same reaction volume and conditions were set for nested PCR using nested primer pair (F2/R2) with products of first PCR as templates after 50 time dilution.

RESULTS

In order to study *in vitro* rapid clonal propagation of indigenous (HSF-240) and exotic (YT-55 & YT-53) cultivars of sugarcane different experiments were conducted. These included surface sterilization, choice of explants and varietals response to different hormonal regimes. Moreover, virus free nature of *in vitro* regenerated plants was validated through RT-PCR analysis.

Complete surface sterilization of field grown explants is achievable: Generally, due to in-built dormant living contaminating entities in sugarcane; it is very arduous to overcome them through surface sterilization. However, in this study complete surface sterilization of selected genotypes (HSF-240, YT-55 & YT-53) was possible when treated with Clorox and cefotaxime at different concentrations and time duration. Without a special note MS basal media with different concentrations of plant growth regulators was used for culture Initiation. Table I shows that %age survival of explants is generally low in case of treatment with 70% Clorox for 10 min. The lowest value is observed for HSF-240, which is only 10%. However, when the concentration of Clorox is increased to 100%, the survival percentage of explants is elevated 2 to 4 times. The exotic varieties (YT-55 & YT-53) exhibit more increase (4 times) in percentage survival than the local ones (2 times). The higher concentration of Clorox was able to control both the fungal and bacterial contaminations. However, it induced browning of explants. Generally, the browning of cultures on MS media was observed on and around the base of the explants due to the release of phenolics. To avoid this exudation, explants were sub-cultured regularly at an interval of 10–15 days by transferring them to the fresh medium of same combination. The dead and brown part of the explants was excised from the base and removed before sub-culturing.

After repeated efforts it was possible to control fungal growth on explants but not the bacterial. In order to control bacterial contamination 500 mg L^{-1} of cefotaxime was added in the media after autoclaving. Remarkably, the effect of cefotaxime was tremendous and 100% control over bacterial contamination was achievable (Table II). Nevertheless, the higher concentration of Clorox and induction of cefotaxime in the media resulted in slower growth of the explants. While comparing the survival percentage of local and exotic varieties, it was observed that the exotic varieties were more responsive to sterilization procedure than local ones. The best survival rate was higher in YT-55 (94%) followed by YT-53 with 92%.

The above data suggested that sterilization protocols for sugarcane could be optimized through the use of high concentration of Clorox and cefotaxime in the media. Hence, complete decontamination of sugarcane explants is possible.

Plant growth regulators, genotype and type of explants affect the culture initiation: Apical meristem and lateral

buds (eyes) were selected for culture initiation on shoot initiation MS media (SI_1, 0.1 mg L^{-1} GA_3; SI_2, 0.1 mg L^{-1} Kn; SI_3, 0.1 mg L^{-1} BAP+0.1 mg L^{-1} Kn + 0.1 mg L^{-1} GA_3+0.1 mg L^{-1} NAA) containing either cytokinin (0.1 mg L^{-1} Kn) or gibberellin (0.1 mg L^{-1} GA_3) or combination of cytokinins, gibberellin and auxin (0.1 mg L^{-1} BAP + 0.1 mg L^{-1} Kn + 0.1 mg L^{-1} GA_3 + 0.1 mg L^{-1} NAA). Table III demonstrates that on the cytokinin supplemented MS media (SI_2); apical buds show 88% shoot initiation frequency, which is the highest. Remarkably, lateral buds located at the basal position of the stem responded better (73%) than top ones with only 46% shoot initiation frequency. On the other hand, in 0.1 mg L^{-1} GA_3 supplemented media (SI_1) shoot induction frequency drastically reduced to 2–3 times. Lateral top explants showed least response (14%) on the MS media supplemented with either of plant hormones e.g. only 14% in case of GA_3 supplemented media. Remarkably, combination of three types of growth regulators (SI_3 media) greatly enhanced the culture initiation frequency and maximum response (96%) was observed in apical buds.

In most cases both the lateral as well as apical buds generated a single shoot. However origin of multiple shoots was also observed in both types of explants (Fig. 1a). But most often it was apical buds that produced multiple shoots. Nevertheless, the shoot developed from lateral buds was weaker and slow growing. By contrast stronger and healthier shoots emerged out of apical buds.

From the above results, it can be inferred that type of explants and plant growth regulators influence the culture initiation. The best suitable combination for high frequency shoot initiation is 0.1 mg L^{-1} BAP+0.1 mg L^{-1} Kn+0.1 mg L^{-1} GA_3+0.1 mg L^{-1} NAA supplemented MS media.

Different varieties show variable responses to plant growth regulators (PGRs) during shoot multiplication: The multiplication of explanting *in vitro* cultures is the crucial step in the propagation of any crop for commercial exploitation, where high frequency and most rapid rate of multiplication are required. For shoot multiplication, MS media supplemented with growth regulatory hormones in two combinations (SM_1: 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} Kn + 0.5 mg L^{-1} GA_3+ 0.1 mg L^{-1} NAA; SM_2: 1 mg L^{-1} BAP + 1 mg L^{-1} Kn + 1 mg L^{-1} GA_3 + 0.25 mg L^{-1} NAA) were used. After 3–4 weeks of shoot initiation, actively growing shoots were shifted to shoot multiplication medium in flasks for further growth and proliferation at rapid rate (Fig. 1). Fig. 2 exhibits that there is a gradual decline in shoot multiplication for all the varieties on SM_1 medium having low concentration of PGRs. By contrast SM_2 medium with higher concentration of PGRs reinforces the multiplication. The average number of shoots on multiplication media SM_1 is 9.5, while it increases to 17.4 on SM_2. Hence, it is almost doubled on SM_2.When varietals response between SM_1 and SM_2 is compared, HSF-240 exhibits maximum multiplication in both the media with 9.5 and 17.4 on SM_1 and SM_2, respectively. Interestingly, local varieties responded better on media with lower concentration of all the PGRs. The lowest number of shoots was observed for YT-53 with 4.6 shoots on SM_1 and 8.5 on SM_2 on the average. It was also observed that the maximum number of shoots was achieved in HSF-240 variety. The shoots were stout and exhibited profuse growth on both the media. On the contrary, in exotic cultures i.e. YT-53 and YT-55, shoots were fragile on SM_1 media in comparison with SM_2. When both media were compared, shoots appeared healthy and elongated at normal rate on SM_2. All the four varieties were

Table I: Effects of different concentrations of Clorox on explants survival

Genotypes	Clorox (%)	Time (Minutes)	Survival (%)
HSF-240	70	20	24
	70	10	15
	100	20	39
	100	10	42
YT-55	70	20	21
	70	10	18
	100	20	63
	100	10	67
YT-53	70	20	17
	70	10	12
	100	20	68
	100	10	72

Different concentrations of Clorox at 70% and 100% for 10 and 20 minutes were used for sterilization of explants (axillary and apical buds). For all the varieties 200 explants were sterilized. Explants survival means no contaminations in the culture and initiation of shoot growth. Values in the left column indicate percentage survival on MS media

Table II: Effect of cefotaxime on the culture initiation

Genotypes	Survival (%)
HSF-240	84
YT-55	94
YT-53	92

Cefotaxime was used in media at a concentration of 500 mg L^{-1}. Explants survival means no contaminations in the culture and initiation of shoot growth

Table III: Effect of explants source, position and PGRs on shoot initiation

Media	PGRs	Axillary basal (%)	Axillary top (%)	Apical (%)
SI_1	GA_3	28	14	34
SI_2	Kn	73	46	88
SI_3	Kn+BAP+GA_3+NAA	85	66	96

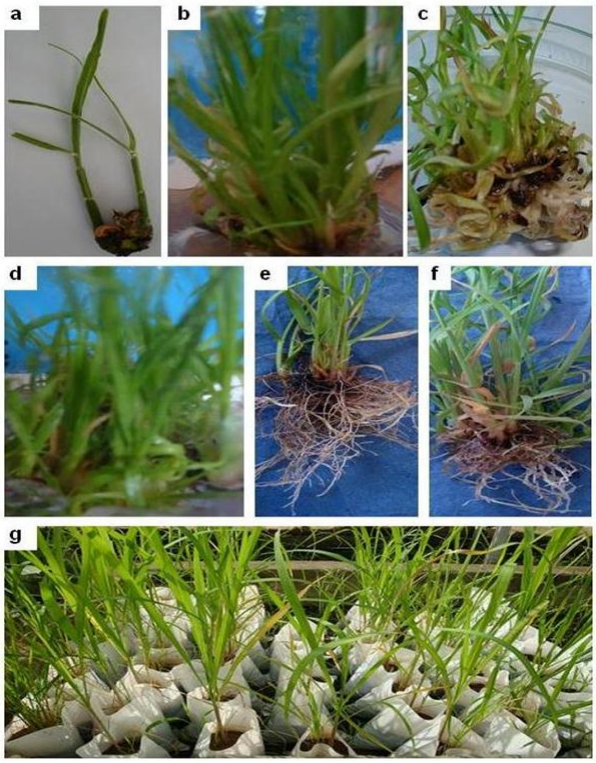
In each case 40 explants were completely sterilized with 500 mg L^{-1} cefotaxime. $SI_{1, 2, 3}$ stands for shoot initiation media 1, 2, 3. SI_1, 0.1 mg L^{-1} GA_3; SI_2, 0.1 mg L^{-1} KINGA; SI_3, 0.1 mg L^{-1} BAP + 0.1 mg L^{-1} Kn + 0.1 mg L^{-1} GA_3 + 0.1 mg L^{-1} NAA. Values show percentage of culture initiation. PGRs, plant growth regulators

Table IV: Hardening off efficiency of *in vitro* raised sugarcane plants

Varieties	Plants shifted to glasshouse	Plants survived in glasshouse	Hardening (%)
HSF-240	213	210	98.6
YT-55	138	132	95.6
YT-53	115	101	87.8

Regenerated plantlets were shifted in polythene bags containing sandy clay loam soil kept in glasshouse

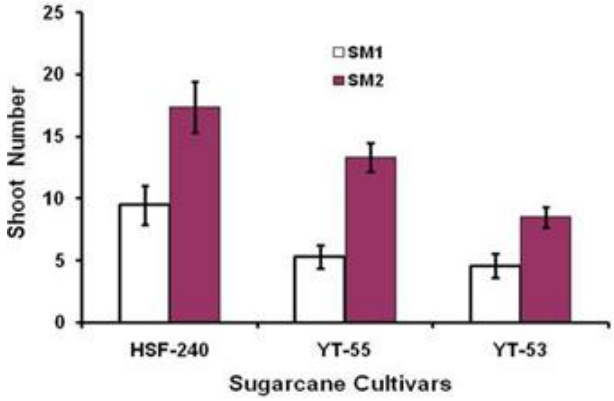
Fig. 1: Micropropagation of sugarcane. (a) apical bud giving rise to multiple shoots; (b) maximum shoot number (9.5 shoots on the average) on SM_1 (0.5 mg L^{-1} BAP + 0.5 mg L^{-1} Kn + 0.5 mg L^{-1} GA_3+ 0.1 mg L^{-1} NAA) exhibited by HSF-240; (c) chlorophyll mutant phenotype (seen as white shoots at the base) observed on SM_1; (d) maximum shoot number (17.4 shoots on the average) shown by HSF-240 on SM_2 (1mgL^{-1} BAP + 1 mg L^{-1} Kn + 1 mg L^{-1} GA_3 + 0.25 mg L^{-1} NAA); (e) best rooting response yielded by HSF-240 on RI_1 (Half strength MS salts + 6% sucrose); (f) best rooting response exhibited by YT-53 on RI_2 (MS + 0.5 mg L^{-1} NAA+ 0.5 mg L^{-1} IAA); (g) acclimatized plants growing in pots in sandy clay loam soil glasshouse



successfully multiplied with enhanced shoot length. It can be inferred that medium containing high quantity of PGRs resulted in high frequency of shoot multiplication and shoot elongation (Fig. 1b–d). After 20 days, these bunches of shoots were excised into bunch of 3–4 shoots and further sub-cultured into fresh medium of same concentration for further propagation. Another phenotype routinely observed was the occurrence of chlorophyll mutants, particularly on SM_1 medium (Fig. 1c).

From the above results, it can be demonstrated that different varieties respond distinctively to the same as well as to different media combination. For shoot multiplication, HSF-240 exhibited the paramount response among all the four varieties. Moreover, HSF-240 proliferated rapidly in both the media combinations. By contrast, exotic varieties i.e. YT-53 and YT-55 were mostly responsive to SM_2.

Fig. 2: *In vitro* shoot multiplication of sugarcane. Different genotypes showing variable responses under different plant growth regulators regimes during shoot multiplication are presented in the form of a bar graph. All the genotypes showed better shoot multiplication frequency on SM_2 media combination with higher concentration of plant regulators where SM stands for shoot multiplication media; SM_1: 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} Kn + 0.5 mg L^{-1} GA_3 + 0.1 mg L^{-1} NAA; SM_2: 1 mg L^{-1} BAP + 1 mg L^{-1} Kn + 1 mg L^{-1} GA_3 + 0.25 mg L^{-1} NAA. Error bars show standard deviation



Therefore, it can be suggested that SM_2 is the best suitable medium for shoot multiplication.

Plant hormones are not essential for profuse rooting: The root induction is the most critical stage as sugarcane plantlets do not often respond well, especially in the later stages (Khan & Rashid, 2003). For root induction two media combinations were employed. These include RI_1: Half strength MS salts + 6% sucrose and RI_2: MS + 0.5 mg L^{-1} NAA+ 0.5 mg L^{-1} IAA. The root induction is often inhibited by cytokinin that is necessary for shoot multiplication (George & Sherrington, 1984). Therefore, for rooting cytokinins were replaced by auxins (NAA & IAA) in the media, which are reported to induce profuse roots. The observations were recorded in the form of root length, root number and number of days for root induction. Fig. 3a exhibits the root length data of three varieties on two different media. The root system developed on root induction media is considered splendid if root length ranges from 8–10 cm from the base of plantlet (Fig. 1e, f). Remarkably, the longest root of 9.2 cm is observed for HSF-240 on RI_1. On the other hand, YT-53 shows only 6.67 cm on RI_1 medium. When two media are compared, RI_2 could not develop an excellent root system. Moreover, the trend for thinner root development was observed. Unexpectedly, the shoot length was increased first in all three varieties before root induction when RI_2 medium was employed. The local variety showed better response than exotic ones. (Fig. 3b, c) demonstrate data on root number per culture and number of days for root induction on the two media. The maximum number of roots induced in HSF-240 is 20.5 in

Fig. 3: Graphs showing root induction in sugarcane. (a) Root length response of different genotypes under two root induction media; RI_1: Half strength MS salts + 6% sucrose; RI_2: MS + 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} IAA. (b) Root number response of different genotypes on two rooting media; RI_1: Half strength MS salts + 6% sucrose; RI_2: MS + 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} IAA. (c) Days to root induction response of different genotypes on two root induction media; RI_1: Half strength MS salts + 6% sucrose; RI_2: MS + 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} IAA. Error bars indicate standard deviation

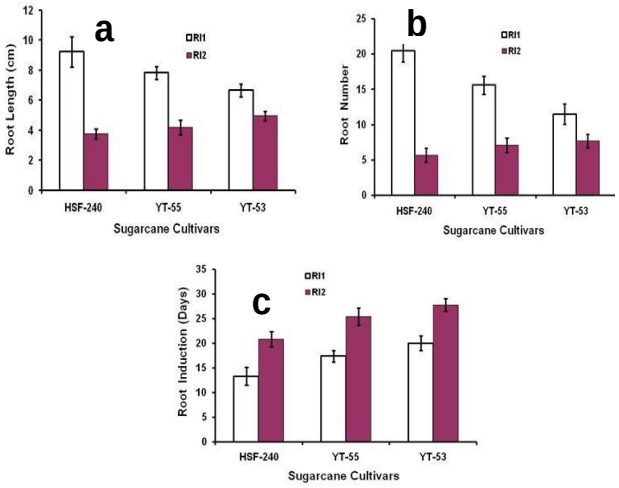
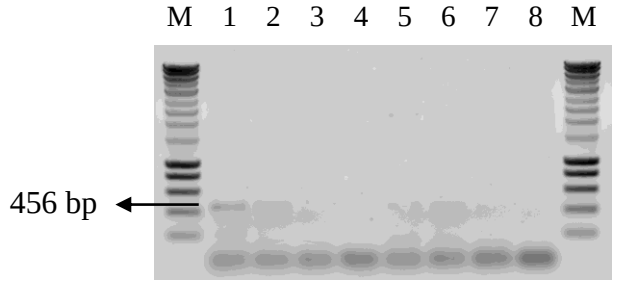


Fig. 4: Two steps RT-PCR analysis of *in vitro* regenerated and infected sugarcane plants for the presence of sugarcane mosaic virus. M: DNA hyper ladder. 1, 2: Infected samples showing presence of SCMV coat protein gene. Arrow indicates 456 bp amplified fragment; 3, 4: *in vitro* regenerated sugarcane samples of YT-55; 5, 6: *in vitro* regenerated sugarcane samples of YT-53; 7, 8: *in vitro* regenerated sugarcane samples of HSF-240



13 days in contrast to 7.7 in YT-53 with 28 days on RI_1 and RI_2, respectively. The trend of thick and vigorous root development was observed in RI_1 compared to RI_2, where root system was not profuse. The slowest root induction was observed in YT-53on both the media. On the contrary, the highest root induction rate was observed in HSF-240, which was 13 and 21 days on RI_1 and RI_2, respectively.

From the above finding, it can be inferred that media with PGRs (NAA & IAA) and without PGR have influence on root length, root number and root induction days. Among all the three varieties tested, HSF-240 yields promising results. Furthermore, PGR-free media is the best supportive media for root induction.

Sandy clay loam soil is the best suitable for acclimatization: After successful rooting, plantlets with profuse roots were transferred to the glasshouse in polythene bags containing two different soil media: sandy clay loam soil and sandy clay loam with peat moss. First attempt was made to acclimatize rooted sugarcane plantlets to peat moss soil. Earlier some plants were shifted in polythene bags containing peat moss soil. Unexpectedly, it was observed that all transplanted plants were failed to survive in peat moss soil and dried within a week. Alternatively, all the acclimatized plantlets with profuse roots showed obvious growth within 5–6 days after they were transplanted in sandy clay loam soil kept in the glasshouse. Later on, all rooted plantlets were acclimatized only in the sandy clay loam soil (Fig. 1g). A total of 56 *in vitro* rooted plants belonging to 213 plants of HSF-240, 138 plants of YT-55 and 115 plants of YT-53 (Table IV) were shifted to glasshouse in polythene bags containing plain soil. Of them, 210, 132 and 101 plants of HSF-240, YT-55 and YT-53, respectively survived successfully. Hardening efficiency ranged from 88–98%. Despite varietal differences, all the three varieties were hardened off with high efficiency. It was observed that only plants with poorly developed root system were failed to survive in plain soil.

From the above results, it is evident that composition of soil media has strong impact on acclimatization of *in vitro* raised plants. Remarkably, HSF-240 showed best hardening efficiency. Moreover, sandy clay loam soil is the best suitable medium for acclimatization of *in vitro* raised sugarcane plants in the glasshouse.

RT-PCR can efficiently be utilized for validation of SCMV: Reverse transcription followed by the polymerase chain reaction (RT-PCR) resulting in amplification of RNA sequence in cDNA form is a sensitive means for detecting RNA molecules transcribed for gene expression. In order to validate the virus-free nature of *in vitro* regenerated plants, two steps RT-PCR analysis for the detection coat protein gene of sugarcane mosaic virus was employed. The cDNA of *in vitro* raised plants as well as of infected field grown plants was used as template to amplify 718 bp PCR fragment with gene specific primers in the first PCR. No PCR products were detectable when resolved on 2% agarose gel. These PCR products were diluted to 50 times with water and 2nd PCR was run with nested set of primers. For the second PCR the amplification of target product with size of 456bp, though weak, was achievable only in the infected leaf samples, while none of the *in vitro* regenerated leaf sample showed any amplification of the target gene (Fig. 4). This indicates that *in vitro* regenerated plants are SCMV free. Hence, the presence of SCMV was

successfully analyzed through two steps RT-PCR. These results clearly demonstrate that RT-PCR can be potentially used for the detection of virus free plants for SCMV.

DISCUSSION

Sugarcane crop plays a vital role in sugar industry and is the source of energy as fuel (Yasmin *et al.,* 2011). For commercial consumption, its need is increasing day by day. Due to land constraints and stagnancy in crop yield, the rapid multiplication of genetically improved elite cultivars through micropropagation is essential. Plant tissue culture has been practiced with great success for efficient clonal propagation of many economical important crops. More than 1.5 million plants can be generated via micropropagation by using shoot tip explants (Anita *et al.,* 2000). The purpose of present study was to contribute development of efficient micropropagation system for the mass multiplication of one indigenous (HSF-240) and two exotic Chinese varieties of sugarcane (YT-55 & YT-53). Furthermore, detection of sugarcane mosaic virus (SCMV) in regenerated and field grown plants using molecular method such as RT-PCR was also the major quest.

Axillary and apical buds were used as explants source since they are found to have high regeneration efficiency (Anita *et al.,* 2000; Roy & Kabir, 2007). The effect of cefotaxime was tremendous and the highest survival percentage recorded was 94%. By analogy, 90% explants growth at 500 mg L^{-1} cefotaxime was reported by Khan *et al*. (2007). Nevertheless, slower growth of the explants in all the genotypes selected was observed. No explants mortality was recorded. By contrast, it was reported that at the higher concentration of two antibiotics; amphotericin (300 µL/mL) and cefotaxime (300 µL/mL) in the culture medium of sugarcane plants, 100% decontamination efficiency was achieved, but the bud explants died indicating that antibiotics became phytotoxic at higher concentrations (Danso *et al.,* 2011).

The highest shoot initiation frequency obtained was 96% when apical bud were employed as explants source on MS media supplemented with four plant growth regulators (0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP + 0.1 mg L^{-1} Kn + 0.1 mg L^{-1} GA_3). On the contrary, 90% shoot tip explants induction frequency was reported when combination of 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA used (Roy & Kabir, 2007). Similarly, Khan *et al.* (2009) achieved 85% shoot growth when 1 mg L^{-1} Kn and 0.1 mg L^{-1} GA_3 supplemented MS media was used. The best suitable media combination for high vigor and shoot multiplication emerged when the concentration of all the four plant regulators (NAA + BAP + Kn + GA_3) were increased to 1 mg L^{-1} except NAA (0.25 mg L^{-1}). Higher concentrations of these PGRs caused considerable changes in morphology of emerged shoots. The shoots not only increased in length but also in vigor. The maximum shoots number recorded was 17.4 on the average. These findings are in corroboration with Roy and Kabir (2007) who obtained 17.2 shoot per culture in media combination containing 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. Previously there exists no report where addition of four PGRs in the same media was reported for shoot emergence and multiplication for sugarcane regeneration. However, it is well documented that different genotypes showed variable responses to same or different media combinations. Besides, these genotypic differences might be due to size and position of explants, tissue juvenility, endogenous level of PGRs and physical growth factors (Pierik, 1997).

Profuse rooting was achievable on half strength MS media along with 6% sucrose without any plant hormones. This means that salt and sugar stress are sufficient to induce rooting in sugarcane. The profuse rooting with 9.2 cm root length and 20.5 roots on average were achievable. In sharp contrast with our results, Khan *et al.* (2008) reported that no rooting was found in media devoid of any plant regulators. A 2nd media with two auxins (0.5 mg L^{-1} NAA + 0.5 mg L^{-1} IAA) tested also induced rooting but developed poor root system (7.7 roots per culture on average). Similar to our findings, Behera and Sahoo (2009) also achieved 13.4 roots per culture on the average with addition of auxin (2.5 mg L^{-1} NAA) in half strength MS media. The inhibition of root induction after the addition of auxin might be due to the deposition of ethylene. The auxins stimulate plant cell to produce ethylene and this resulted in retardation of root elongation (Weiler, 1984; Pau & Chi, 1993; Ali *et al.,* 2008). Hardening efficiency of *in vitro* regenerated sugarcane plants on sandy clay loam soil under glasshouse was tremendous (98%) in comparison with 95% and 85% as reported by Gill *et al.* (2006) and Behera and Sahoo (2009), respectively.

When plant material undergoes vigorous multiplication like during *in vitro* micropropagation, the viruses in the explants hide themselves and do not appear during the active growth of explants. But when plants are moved to glasshouse for hardening purpose; virus might appear and shows its symptoms in the plants. Therefore, it is important to test the virus free nature of tissue culture plants for achieving virus free planting material for further propagation. Sugarcane mosaic virus belongs to the viral family Potyviridea and recently classified in the SCMV subgroup based on coat protein region and genomic region (Shukla *et al.,* 1989; 1992; Frenkel *et al.,* 1991). Its importance can be recognized as it severely affects the sugarcane crop throughout the world. Moreover it also damages cash crop of the Poaceae family like maize, sorghum and other wild grass species (Mohammadi & Hajieghrari, 2009). SCMV has genetic storage material as single stranded positive sense RNA and it is rod shaped virus. It is frequently transmitted by three modes; aphids, infected propagating material and any mechanical source. The mechanical transmission mostly affects when plants are under glasshouse and during laboratory study while other

two modes affect plants in the fields (Mohammadi & Hajieghrari, 2009).

Recently, the application of the RT-PCR technique has resulted in clear and fully reproducible method for diagnostic assay of virus infected leaf material. This is well ensured for its eventual application as a reliable and definitive test for SCMV regardless of strain. Work is in progress to establish how universally the test can be applied for the diagnosis of SCMV. Hence, to validate virus free nature of *in vitro* regenerated sugarcane plants through molecular method, two steps RT-PCR was employed. RT-PCR has some advantages over other serological analysis like enzyme linked immunosorbent assay (ELISA) because it requires only small amount of samples and full analysis including PCR can be done in less than 24 h (Huckett & Botha, 1996). Specific amplification of target product depends on selection and designing of specific primers (Xie *et al.,* 2009). Hence, the primer pairs used in the present study were designed to match all sequences of SCMV available in GeneBank. It has the potential to detect most of the variants of SCMV, if not all. Therefore, in the present study presence of SCMV was successfully analyzed through two steps RT-PCR. The result revealed successful validation the virus free nature of the *in vitro* raised sugarcane plants.

In the conclusion, the protocols developed here have provided efficient approaches not only for *in vitro* multiplication of sugarcane elite genotypes directly through apical and axillary buds but also for detection of virus-free nature of regenerated plantlets through RT-PCR. It is confirmed that PGRs work better in combinations than singly. Moreover, PGRs are not necessary for root establishment of sugarcane plants. Our studies can be efficiently utilized for mass propagation of elite sugarcane cultivars for commercial exploitation and for the genetic transformation of any trait. This methodology can also be extended to other crops of economic interest not manipulated yet.

From the results, it can be concluded that complete sterilization of explants is achievable and cefotaxime @ 500 mg L^{-1} is optimum to control bacterial contaminants. The response of shoot induction and multiplication is PGRs and genotype dependent. Furthermore, PGRs are more effective in combinations. PGRs-free medium was best suitable for root induction. Most importantly, RT-PCR is a reliable assay for detection of virus free nature of *in vitro* regenerated sugarcane plants.

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